68. A method for preventing thrombus formation in an animal or human, comprising administering to the animal or human a composition comprising polynitrosated hemoglobin in a therapeutically effective amount.

69. A method for preventing thrombus formation in an animal or human, comprising administering to the animal or human a composition comprising SNO-Hb[Fe(II)] in a therapeutically effective amount.

70. A method for inhibiting platelet activation in a mammal, comprising administering to the mammal a composition comprising SNO-hemoglobin in a therapeutically effective amount.

71. A method for inhibiting platelet activation in a mammal, comprising administering to the mammal a composition comprising SNO-methemoglobin in a therapeutically effective amount.

REMARKS

Claims 50, 52, 54, 55, 57 and 58 have been canceled. Claims 64-71 have been added. Claims 64, 65, 66 and 68 are (the now-canceled) Claims 50, 52, 54 and 57, respectively, redrafted in independent form. Claims 67 and 69 have been redrafted in independent form from (now-canceled) Claims 55 and 58, respectively, and have been amended to substitute SNO-Hb[Fe(II)] for methemoglobin. Claims 70 and 71 are analogous to Claim 59; for support see page 34, lines 14-25, and as referred to therein, Example 9 and Figures 7A-7C.

For support of claims to methods using SNO-hemoglobins (Claims 15-17, 59 and 64-71), refer to page 34, line 14 to page 36, line 26, for example. For support of claims to methods of using nitrosylhemoglobin (Claims 15-17 and 59-63), refer to page 39, line 15 to page 41, line 23, for example.

Definitions of "Nitrosated Hemoglobin" and "Nitrated Hemoglobin"

A definition to be used in interpreting the claims reciting "nitrosated hemoglobin" appears on page 37, lines 1-12. It should be seen from this definition that S-nitrosohemoglobin, (SNO-hemoglobin), nitrosylhemoglobin (having NO bound to the heme Fe), and polynitrosated hemoglobin are all within this definition. S-nitrosohemoglobin is also referred to as S-nitrosylhemoglobin, SNO-hemoglobin or SNO-Hb, and can be in the forms of SNO-Hb[Fe(II)] (also written as SNO-deoxyHb), SNO[Fe(II)]O_2 (also written as SNO-oxyHb), or SNO[Fe(III)] (also written as SNO-metHb or SNO-methemoglobin). Polynitrosated hemoglobin has NO adducts at additional sites (*e.g.*, O, N, or possibly C atoms) besides both sulfur atoms of the β93 cysteines, and therefore, can also be considered a form of S-nitrosohemoglobin. One of ordinary skill in the art would predict this to be the result of the order of the reactivity of the nucleophilic sites of the hemoglobin molecule, known from general chemical principles: S > O > N > C. Thus, a nitrosated hemoglobin has at a minimum, an NO group bound at a heme Fe or at a sulfur atom derived from a thiol group.

The term "nitrated hemoglobin" is not given any special definition in the written description. Thus, it will be understood, from the meaning of "nitrate" in chemistry, that "nitrated hemoglobin" is a species of hemoglobin having one or more nitrate groups (-NO_2). It will also be understood by one of ordinary skill in the art that agents facilitating nitrosations, nitrosylations or nitrations will result in the attachment of NO_x groups, depending on the attachment site and conditions. See the written description at page 37, lines 23-28.

Nitrosated Hemoglobins -- Teachings of the Prior Art and Teachings of the Subject Application

Nitrosylhemoglobin (NO bound at one or more heme Fe atoms) was known in the prior art. It was known that the affinity of NO for the heme Fe is extremely high and that the reaction in which nitrosylhemoglobin dissociates to NO and unbound hemoglobin has an extremely low rate, so low that the binding of NO to the Fe of hemoglobin can be considered essentially irreversible. For a useful comparison of affinities of NO, CO and O_2 to the heme Fe, see Greenburg, A.G. and H.W. Kim, *Art. Cells, Blood Subs., and Immob. Biotech. 23*:271-276, 1995, especially fifth paragraph on page 272 (reference AX), wherein it is said that the affinity of NO

for the heme Fe is 1,000 times that of CO and 200,000 times that of O_2. For k_{on} and k_{off} rates for NO in the reaction NO + hemoglobin ↔ nitrosylhemoglobin, see reference AY4, Kharitonov *et al.*, pp. 39-45 in *Methods in Nitric Oxide Research*, Feelisch and Stamler, eds., John Wiley and Sons, Ltd., 1996, especially Table 2, page 41, from which it can be seen that the dissociation constant for NO from nitrosylhemoglobin is on the order of 10^{-12}. The other forms of nitrosated hemoglobins were unknown before the work described in this application and the priority applications.

This application describes the synthesis and physiological effects of new species of hemoglobin unknown in the prior art (S-nitrosohemoglobins), and shows that S-nitrosohemoglobin is an endogenous species of hemoglobin present at low concentrations as a normal constituent of hemoglobin in the red blood cells (see Example 8 in the written description, page 76, line 1 to page 77, line 9). The properties of nitrosated hemoglobins and the use of nitrosated hemoglobins in methods of therapy for anti-platelet effects are discussed in the written description at page 34, line 14 to page 36, line 26, for instance. The application also describes a previously unknown intramolecular reaction in which nitrosylhemoglobin is converted, through an intramolecular reaction that occurs at physiological conditions, to S-nitrosohemoglobin. Thus, at physiological conditions *in vivo*, nitrosylhemoglobin can be converted to S-nitrosohemoglobin, and therefore, can be useful in methods of therapy as a donor of NO. See, *e.g.*, Examples 18 and 19, on page 86, line 9 to page 87, line 11 of the written description. See also page 39, line 27 to page 41, line 23.

Additional Comments Regarding Cited Reference Stamler *et al.* (WO 93/09806) Applicable to Rejections Under 35 U.S.C. § 103(a) in Items 2, 3 and 4 of Office Action of 15 August 2000

From the teachings of the prior art one of ordinary skill in the art might conclude that it is desirable to administer a donor of NO to inhibit platelet function in blood clotting. See the written description at page 3, lines 26-30, page 4, lines 4-22 and page 33, line 4 to page 34, line 13, for a discussion of the prior art.

The logic behind the experiments described in WO 93/09806 that one of ordinary skill in the art might conclude is that because proteins contain thiol groups (at cysteine residues), S-

nitrosylated proteins might be used as NO donors, just as some low molecular weight S-nitrosothiols were already known to be NO donors.

Several proteins which were readily available in purified form (tissue plasminogen activator, bovine serum albumin, cathepsin B, lipoprotein and immunoglobulin) were used as substrates in nitrosylation reactions with acidified nitrite as the reagent.

An attempt was made (described in Example 19 of WO 93/09806) to produce a similar reaction using hemoglobin. However, at that time, the choice to include hemoglobin as one of the proteins that could possibly act as a donor of NO was somewhat illogical. It had been generally thought that nitric oxide reacted with hemoglobin in two major ways: 1) with the deoxyhemoglobin to form a stable nitrosyl (FeII) heme adduct (nitrosylhemoglobin); and 2) with oxyhemoglobin to form nitrate and methemoglobin – a reaction that inactivates NO. These two reactions contributed to the idea that hemoglobin is a scavenger of NO (Wennmalm *et al., Br. J. Pharmacol. 106*:509-510, 1992; reference AS5). In both of these reactions, NO biological activity is lost. Thus, it was somewhat illogical to think that nitrosated hemoglobin, should it be possible to make, would act as a nitric oxide donor like low molecular weight S-nitrosothiols, when the prior art indicated that any NO in the vicinity of the heme Fe would be bound by the heme Fe and inactivated by one or the other mechanism.

No nitrosohemoglobin was produced by any methods described in WO 93/09806. It was demonstrated by experimental evidence accompanying the Declaration of Jonathan S. Stamler, M.D. Under 37 C.F.R. § 1.132 mailed to the Patent Office on September 2, 1999, that no S-nitrosohemoglobin was produced in an attempt to reproduce the experimental conditions presented in Example 19 on pages 58-59 of WO 93/09806. Because no S-nitrosohemoglobin could be made by this method, it is most reasonable to conclude that NO adducts did not result at O, N, or C sites on hemoglobin by this method. The thiol groups of the hemoglobin molecule are more reactive nucleophilic sites than O, N or C sites. The product made by the method of Example 19 is dissociated subunits of hemoglobin, with the heme iron showing a spectrum characteristic of methemoglobin. See the spectra in Figure 29 of WO 93/09806, and item 6 on page 4 of the Declaration of Jonathan S. Stamler, M.D. Under 37 C.F.R. § 1.132 mailed to the United States Patent and Trademark Office on 2 September 1999.

The subject application and priority applications describe a further finding which would be unexpected to one of ordinary skill in the art at the time of the invention. Although SNO-hemoglobin in the deoxy and met forms are vasodilators, as would be expected of NO donors, the oxy form of SNO-hemoglobin, when administered alone, can act as a vasoconstrictor, as demonstrated in standard bioassay systems. See in the written description Example 4, especially page 68, line 3 to line 14, and Figure 4A, and Example 25, especially page 98, lines 25-29.

Nitrosylhemoglobin was only briefly mentioned in the Stamler *et al.* WO 93/09806 reference, on page 58, line 21. In WO 93/09806, and in all other prior art known to applicants, nitrosylhemoglobin was never discussed as being, or possibly being, a donor of NO, nor is any reaction described by which nitrosylhemoglobin can be converted to S-nitrosohemoglobin or anything else that could act as a donor of NO. Polynitrosated hemoglobin species were not discussed at all in WO 93/09806.

Additional Comments Regarding Cited Reference Kaesemeyer (US 5,543,430) Applicable to Rejections Under 35 U.S.C. § 103(a) in Items 2, 3, and 4 of Office Action of 15 August 2000

The Kaesemeyer reference (US 5,543,430) has been cited for its teaching of nitrates being donors of NO. The Examiner states the reasoning:

> Accordingly, it would have been obvious to one of ordinary skill in the art at the time of applicant's invention to substitute nitrated hemoglobins for nitrosohemoglobins in the methods as disclosed by Stamler with a reasonable expectation of success due to functional equivalency of nitrates as NO donating compounds.

Other findings known in the prior art would lead one of ordinary skill in the art to a different expectation. It was known that hemoglobin scavenges NO, thereby producing nitrosylhemoglobin, which is extremely stable according to the prior art. On that teaching alone, one would not expect nitrated hemoglobin to act as a donor of NO, for if nitrate could be released from nitrated hemoglobin as some biologically active form of NO, it would be expected, according to the teaching of the prior art, that the same hemoglobin molecule or another hemoglobin molecule in the vicinity would bind the NO at the heme, thereby producing nitrosylhemoglobin, or alternatively, producing methemoglobin and inorganic nitrate.

A second scientific finding in the prior art also contradicts the expectation recited by the Examiner. That is, that nitrated hemoglobin (and any other form of hemoglobin) does not pass into the cells. It was known that low molecular weight nitrates such as those discussed in Kaesemeyer must first enter the tissues to be metabolized to a biologically active form of NO.

INTERVIEW SUMMARY

The subject application and its immediate parent application, 08/796,164, were discussed in a telephonic interview with Examiner Celsa on February 13, 2001, with Applicant Jonathan S. Stamler and the undersigned attorney, Carol A. Egner, participating. A summary of the discussion with respect to the subject application appears below.

The terms defining the various forms of hemoglobin and what each form is were discussed. In particular, the scope of the term "nitrosated hemoglobin" was discussed. The definition for this term appearing on page 37, lines 1-12, was pointed out, and is further elaborated upon elsewhere in this paper.

Relevant teachings about the forms of hemoglobin known in the prior art were summarized for the Examiner. Nitrosylhemoglobin was well known and characterized in the prior art, as can be seen, for example, by two references cited in the Office Action of August 15, 2000; Moore *et al., J. Biol. Chem. 251*:2788-2794 and Sharma *et al., J. Biol. Chem. 253*:6467-72. The extremely low dissociation constants for nitrosylhemoglobin given in these cited references and in other references provide an indication of the stability of the NO-Fe bond.

The physiological effects of administering purified, unmodified hemoglobin to a human or other mammal were brought up. Hemoglobin is known from prior art studies to act as a scavenger of NO, effectively binding the NO irreversibly. Hemoglobin produces a vasoconstrictive effect, and activates platelets in the blood clotting process. In some cases, the effects of administering hemoglobin can be fatal.

The new findings described in the patent application and the inventions arising from them were summarized for the Examiner. The properties of SNO-hemoglobins, which can be used as NO donors, and the properties of nitrosylhemoglobin, which can be converted *in vivo* to SNO-

hemoglobin and can produce the same physiological effects, were described. These properties are further developed elsewhere in this paper.

The teachings of the Wade and Castro reference were discussed (Wade and Castro, *Chem. Res. Tox.* 3:289-291, 1990). It was presented to the Examiner that the reaction described in this paper included as reactants not only hemoglobin and NO, but also a small organic nucleophile. The products were the nitrosylated small organic nucleophile (phenol nitrosylated at O, acetylcysteine nitrosylated at N, or proline nitrosylated at N) and nitrosylhemoglobin, with NO bound at the heme Fe. NO was not reported to be, or speculated to be, at any other site in hemoglobin.

The teachings of the Kaesemeyer patent (US 5,543,430) were also discussed, as summarized elsewhere herein.

The Stamler WO 93/09806 reference, cited by the Examiner in the three obviousness rejections in the Office Action of August 15, 2000, was also discussed. Based on the Declaration of Jonathan S. Stamler, M.D. Under 37 C.F.R. § 1.132 filed on 2 September 1999 and exhibits accompanying the Declaration, the Examiner stated that he has accepted that WO 93/09806 does not present an enabling description of a method to produce S-nitrosohemoglobin, and that S-nitrosohemoglobin was not produced at that time. There remained a question of whether other species of nitrosated hemoglobin could have been produced from methods described in WO 93/09806. It was presented to the Examiner that it would be very unlikely that NO adducts were produced at O, N or C atoms in the hemoglobin molecule, if none could be detected at S atoms, because the thiol groups were the most reactive nucleophilic sites. The product of the method used on hemoglobin was oxidized at the heme (methemoglobin). WO 93/09806 does not present a method to produce nitrosylhemoglobin, and before the invention by Applicants, nitrosylhemoglobin would not have been expected to be, or to be converted to, a donor of NO.

It was emphasized that hemoglobin is very unlike the other proteins described in WO 93/09806, because of its property of binding NO. It was also emphasized that once SNO-hemoglobin was produced by Applicants, it was not in all cases a vasodilator, but can be a vasoconstrictor in the oxy form. One of ordinary skill could not have predicted this effect from the teachings of WO 93/09806.

CONCLUSION

The Examiner is respectfully requested to consider the above amendments and remarks made in response to the rejections, and to reconsider the application. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,

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